Asia Pacific Journal of Molecular Biology and Biotechnology, 2003
Vol. 11 (1) : 43-50

Fluorescent Amplified Fragment Length Polymorphism and Pulsed-Field Gel Electrophoresis Analyses of Multidrug Resistant *Salmonella enterica* serotype Typhi from different geographical endemic regions in Asia

Thong Kwai Lin [1]*, Sleman Elgar[1], Zulfikar Bhutta [2],
Rohani Yasin[3] and Edgar Schreiber[4]

[1]*Institute of Biological Sciences, Faculty of Science, University of Malaya, Kuala Lumpur, Malaysia*
[2]*Aga Khan Uniersity, Medical Centre, Karachi, Pakistan*
[3]*Institute for Medical Research, Malaysia,* [4]*PE Biosystem, Foster City, USA*

Received 22 November 2002 / Accepted 2 June 2003

Abstract. Sixty-three isolates of *Salmonella enterica* serotype Typhi (*S.typhi*) from sporadic cases of typhoid fever obtained from Malaysia (n=6), Vietnam (n=13), India (n=8) and Pakistan (n=36) were characterized by phage typing, drug-susceptibility testing, amplified fragment length polymorphism (AFLP) and pulsed-field gel electrophoresis (PFGE) with restriction endonuclease, *Xba*I. The strains analyzed were mostly resistant to ampicillin, co-trimoxazole, tetracycline and chloramphenicol. These strains were represented by phage types, E1, E9, 46, J1, VNS and UVS. PFGE analysis showed that multidrug resistant (MDR) strains isolated in different countries and at different periods were genetically very homogenous where 90% of the strains analyzed had very closely related *Xba*I patterns. AFLP was able to subtype 42 clonally related MDR strains (represented by 8 PFGE patterns) into 16 profiles. Cluster analysis based on the AFLP and PFGE data using unweighted pair group mean averages could differentiate MDR strains from different geographical region and that the drug-sensitive strains were in a distinct cluster. The clustering of MDR strains from each country and the presence of a dominant *Xba*I–PFGE pattern indicated that the MDR *S.typhi* had probably been spread clonally in these countries. AFLP is clearly more discriminative than PFGE in differentiating the MDR *S.typhi,* hence providing an alternative, sensitive method for detailed analysis of the multidrug resistant strains.

Keywords. AFLP, PFGE, *S. typhi*

INTRODUCTION

With regard to emerging infectious diseases, typhoid fever caused by multidrug resistant (MDR) strains of *Salmonella enterica* serotype Typhi (herein named as *S. typhi*) present a serious health problem, especially in the Asian and Southeast Asian countries. Multidrug resistant (MDR) *S. typhi* strains were defined as those resistant to ampicillin, chloramphenicol and trimethoprim-sulphamethoxazole with additional resistance to streptomycin, sulfonamides and tetracycline. Drug-resistant *S.typhi* began to appear in the late 1980's in the South Asian region and have now spread widely to the Middle East, Africa and Asia (Rowe *et. al.*, 1997). The problem is particularly acute on the Indian subcontinent where approximately 60-65 % of strains isolated are MDR strains. In addition, the disease associated with MDR tends to be more severe with patients registering a higher morbidity score and a higher mortality rate, often with unusual complications (Bhuttta, 1996). The impact of this MDR further complicates the antibiotic treatment by limiting treatment options, prolonged hospitalization and the increasing costs of therapy. In many tropical countries, typhoid fever is endemic and has the potential for epidemic spread as drug resistance is often encoded by plasmids and are highly transmissible (Connerton *et al.*, 2000). Studies have shown these plasmids encoding resistance to chloramphenicol, ampicillin, trimethoprim, sulfonamides, and tetracycline belonged to the IncHI incompatibililty group and are conjugative (Hermans *et al.*, 1996, Hampton *et al.*, 1998).

The ability to characterize and subtype *S. typhi* isolates is important from an epidemiological perspective in order to trace and control the dissemination of this infectious agent. Recently, many DNA-based approaches have been utilized in the molecular subtyping of S. *typhi*, including ribotyping (Ling *et al.*, 2000), pulsed-field gel electrophoresis (PFGE)

*Author for Correspondence.
Institute of Biological Sciences, Faculty of Science, University of Malaya, 50603, Kuala Lumpur, Malaysia. Tel: 603-79674437; Fax: 603-79675908; Email: thongkl@um.edu.my

(Thong *et al.,* 2002), IS200 (Navarro *et al*., 1996), RAPD (Shangkuan and Lin, 1998) and amplified fragment length polymorphism (AFLP) (Nair *et al.,* 2000). MDR *S.typhi* from Vellore, India (Shanahan *et al*., 1998), Bangladesh (Hermans *et al*., 1996), Pakistan (Thong *et al*., 2000) and Vietnam (Connerton *et al.,* 2000) showed limited genetic diversity. Our previous study on a hundred strains of MDR and drug-sensitive strains of *S. typhi* from Pakistan demonstrated that the MDR strains were genetically homologous and co-existed with the antibiotic sensitive strains as distinct, independent clones (Thong *et al*., 2000). In the present study, we extend the PFGE analysis to include more drug-resistant *S. typhi* strains from different endemic countries in Asia with the objective of assessing the prevalence of any particular PFGE subtype (s).

A more recent method of amplified fragment length polymorphism (AFLP), a high-resolution PCR-based technique has been reported to be more discriminative and is able to differentiate strains of bacteria which were highly related or identical by another typing methods (Janssen *et al*., 1996, Aarts *et al*., 1998). AFLP technique involves the digestion of total genomic DNA with restriction enzymes and compatible oligonucleotide adapters are ligated to the ends of the resulting DNA fragments. Specific subsets of DNA cleavage products are then amplified from the pool of restriction fragments using various combinations of selective primers. The resulting amplified products are then separated on a DNA sequencing gel. AFLP has been used in the genotyping of several pathogenic bacteria including *Salmonella* spp (Aarts *et al*., 1998), *S. typhimurium* (Tamada *et al.,* 2001) and drug sensitive *S.typhi* (Nair *et al.,* 2000). In this report, we compare the application of AFLP in subtyping of MDR and drug-sensitive *S. typhi* from Pakistan, India, Malaysia and Vietnam with PFGE analysis. Two questions were addressed: (1) Is AFLP able to discriminate MDR strains of *S. typhi* which appear to be clonally related by PFGE ? (2) Is AFLP able to discriminative *S. typhi* from various geographic regions?

MATERIALS AND METHODS

Bacterial strains. A total of 63 human isolates obtained from sporadic cases of typhoid fever obtained from Malaysia (n=6), Pakistan (25 MDR, 11 drug-susceptible), India (n = 8), Vietnam (n =13) were used in this study. Strains were isolated and identified using standard biochemical methods and serotyped using the Kauffmann-White scheme at the microbiology laboratories in the countries of origin. Phage typing of the strains was performed at the Salmonella Reference Center, Institute of Medical Research Kuala Lumpur. Except for the Indian strains (blood cultures), all the other strains were isolated from stool cultures.

Antimicrobial susceptibility tests. The Kirby-Bauer disc-diffusion method (1966) was performed on Muller-Hinton agar with Oxoid disks (Oxoid, Hampshire,UK) according to the instruction of the manufacturer. Antibiotic concentrations (μg) in disks were ampicilin 10(Amp), chloramphenicol 30 (Cm), gentamicin 5 (G), tetracycline 30 (T), nalidixic acid 30 (Nx), kanamycin 30 (Km), trimethoprim-sulphonamethoxazole 25 (Tm-Su), ceftriaxone and streptomycin 10 (Sm).

AFLP fingerprinting. Genomic DNA for AFLP analysis was prepared by the modified method of Saito and Miura (1963). The quantity of genomic DNA of each strain was adjusted by the spectrophometric method (OD_{260nm}) to about 10μg. AFLP fingerprinting was performed essentially as described by the manufacturer (PE Applied Biosystems, Foster City, CA, USA). Genomic DNA was digested with *Eco*RI and *Mse*I and ligated with *Eco*RI and *Mse*I AFLP adaptor pairs (PE Applied Biosystems, Foster City, CA, USA) using T4 DNA ligase. These serve as unique primer binding sites for subsequent PCR amplification. Using the AFLP™ Microbial Fingerprinting Kit (PE Applied Biosystems, Foster City, CA, USA), subsets of genomic DNA fragments with three distinct fluorescent endlabels are generated in subsequent PCR amplification. The resultant AFLP fragments were electrophoresed along with an internal size standard (GeneScan OX-500 size standard, PE Applied Biosystems) on a PE ABI Prism on a 310 instrument using parameters supplied by the manufacturer. Fragment patterns were then analyzed using a ABI Prism Gene Scan Software. An inventory of all peaks /fragments ranging from 50-500bp was generated by using the Gene Scan System. The binary data (electropherogram) were then analyzed further using the phylogenetic analysis program PAUP*4 (Sinauer Associate, Cambridge, MA, USA) and computed using parsimony or distance analysis (UPGMA) or neighbor joining algorithms.

PFGE fingerprinting. Intact, chromosomal DNA for PFGE analysis was prepared in agarose using the modified protocol as previously described (Thong and Pang, 1996). PFGE of *Xba*I digested chromosomal DNA was carried out on a CHEF-DR II/III system for 26 hours at 6V/cm with a ramped pulsed time of 1 to 40 sec. Lambda DNA concatemer PFG marker was used as a DNA size standard. Fingerprinting profiles were examined visually as well as by GelCompar software (Version II, Applied Maths, Kortirijk, Belgium). The presence and absence of a band was scored and strains that differed by one band were assigned different pulse-field profiles (PFPs). The preparation of DNA from the strains was repeated, digested and electrophoresed on three occasions to assess the reproducibility and stability of PFGE method. The objective measure of similarity/dissimilarity between strains was the F values (coefficient of similarity), given by the formula: $F= 2n_{xy}/(n_x + n_y)$ where n_x = number

Table 1. Phage types, resistance phenotypes, *Xba*I-PFGE and AFLP profiles of drug-resistant and drug-sensitive *Salmonella enterica* serotype Typhi strains from different geographical endemic regions in Asia (all clinical isolates from sporadic cases of typhoid fever).

Strain No	Country of origin (year of isolation)	Phage type	Resistance phenotypes	*Xba*I-PFGE type (n= 63)	AFLP type (n= 53)
TP12	Malaysia (1997)	E1	Cm/Tm-Su/Amp	X1	A2a
TP28	Malaysia (1997)	E1	Cm/Tm-Su/Amp	X1	A1a
TP30	Malaysia (1997)	VNS	Cm/Tm-Su/Amp	X1a	A5
TP170	Malaysia (1997)	E9	Cm/Tm-Su/Amp	X1c	nd
TP26	Malaysia (1997)	E1	Cm/Tm-Su/Amp	X1d	nd
M633	Malaysia (1992)	E1	Cm/Tm-Su/Amp/Te/Km	X4	nd
Vn70	Vietnam (1996)	E1	Cm/Tm-Su/Tet/Amp	X1	A1
Vn109	Vietnam (1997)	E1	CmTm-Su/Tet/Amp	X1	A5c
Vn138	Vietnam (1996)	UVS	Cm/Tm-Su/Tet/Amp	X1	A5a
Vn139	Vietnam (1997)	E1	Cm/Tm-Su/Tet/Amp	X1	A5a
Vn229	Vietnam (1997)	E1	Cm/Tm-Su/Tet/Amp	X1	A5b
Vn181	Vietnam (1996)	E1	Cm/Tm-Su/Tet/Amp	X1	A1
Vn357	Vietnam (1996)	UVS	Cm/Tm-Su/Tet/Amp	X1	A1
Vn97	Vietnam (1997)	UVS	Cm/Tm-Su/Tet/Amp	X1a	nd
Vn99	Vietnam (1995)	UVS	Cm/Tm-Su/Tet/Amp	X1b	nd
Vn137	Vietnam (1996)	UVS	Cm/Tm-Su/Tet/Amp	X1	nd
Vn146	Vietnam (1996)	UVS	Cm/Tm-Su/Tet/Amp	X1	nd
Vn187	Vietnam (1996)	UVS	Cm/Tm-Su/Tet/Amp	X1	nd
Vn243	Vietnam (1996)	UVS2	Cm/Tm-Su/Tet/Amp	X2	nd
S5	India (1995)	VNS	Cm/Tm-Su	X1	A6
S15	India (1995)	E1	Cm/Tm-Su/Cef	X1	A1
S23	India (1995)	VNS	Cm/Tm-Su	X1	A1
S40	India (1995)	VNS	Tm-Su	X1b	A6b
S43	India (1995)	VNS	Cm/Tm-Su	X3	A6a
S45	India (1995)	E1	Cm/Tm-Su	X1	A11
S47	India (1995)	E1	Cm/Tm-Su	X2	A6a
S51	India (1995)	UVS4	Cm/Tm-Su	X1	A11
PR1	Pakistan (1997)	VNS	Cm/Tm-Su/Amp/Tet	X1	A1a
PR2	Pakistan (1997)	UVS	Cm/Tm-Su/Amp/Tet	X1	A1
PR4	Pakistan (1997)	UVS	Cm/Tm-Su/Amp/Tet	X1	A7a
PR5	Pakistan (1997)	E1	Cm/Tm-Su/Amp/Tet	X1	A1
PR7	Pakistan (1997)	UVS	Cm/Tm-Su/Amp/Tet	X1	A1
PR8	Pakistan (1997)	UVS	Cm/Tm-Su/Amp/Tet	X1	A1
PR11	Pakistan (1997)	E1	Cm/Tm-Su/Amp/Tet	X1	A1
PR12	Pakistan (1997)	E1	Cm/Tm-Su/Amp/Tet	X1	A7a
PR14	Pakistan (1997)	E1	Cm/Tm-Su/Amp/Tet	X1	A1
PR15	Pakistan (1997)	E1	Cm/Tm-Su/Amp/Tet	X1	A7
PR18	Pakistan (1997)	E1	Cm/Tm-Su/Amp/Tet	X1	A1
PR20	Pakistan (1997	E1	Cm/Tm-Su/Amp/Tet	X1b	nd
PR22	Pakistan (1997)	UVS	Cm/Tm-Su/Amp/Tet	X1	A1
PR23	Pakistan (1997)	E1	Cm/Tm-Su/Amp/Tet	X1	A1
PR25	Pakistan (1997)	E1	Cm/Tm-Su/Amp/Tet	X1	A1b
PR26	Pakistan (1997)	E1	Cm/Tm-Su/Amp/Tet	X1	A1b
PR27	Pakistan (1997)	E1	Cm/Tm-Su/Amp/Tet	X1	A1
PR28	Pakistan (1997)	E1	Cm/Tm-Su/Amp/Tet	X1	A1
PR32	Pakistan (1997)	E1	Cm/Tm-Su/Amp/Tet	X1	A8
PR35	Pakistan (1997)	E1	Cm/Tm-Su/Amp/Tet	X1	A1
PR36	Pakistan (1997)	E1	Cm/Tm-Su/Amp/Tet	X1	A8
PR41	Pakistan (1997)	E1	Cm/Tm-Su/Amp/Tet	X1	A1
PR47	Pakistan (1997)	E1	Cm/Tm-Su/Amp/Tet	X1	A9a
PR48	Pakistan (1997)	E1	Cm/Tm-Su/Amp/Tet	X1	A9a
PR49	Pakistan (1997)	E1	Cm/Tm-Su/Amp/Tet	X1	A9
PS51	Pakistan (1997)	J1	sensitive	X5	A2a
PS52	Pakistan (1997)	J1	sensitive	X5	A10
PS53	Pakistan (1997)	UVS	sensitive	X6	A2
PS56	Pakistan (1997)	46	sensitive	X7	A3

nd=not determined; Cm=chloramphenicol; Tm-Su=trimethoprim-sulfametaxazole; Amp=ampicilin; Cef= ceftrixone; Tet= tetracycline; km=kanamycin

of fragments for isolate x, n_y = number of fragments for isolate y. The quantitative measure of the discriminatory ability of each technique is calculated based on the Simpson Index of Diversity (DI) (Hunter and Gaston, 1988).

RESULTS

Phage types. Eight phage types, E1, E9, 46, J1, VNS, UVS, UVS2 and UVS4 were noted among all the strains. The most common phage type among the MDR *S.typhi* analyzed was E1 (60 %) (Table 1).

Resistance phenotypes. Most of the strains tested were resistant to ampicillin (Amp), chloramphenicol (Cm) and trimethoprim-sulfamethoxazole (Tm-Su) and tetracycline (Tet). Indian strains were mostly resistant to Cm and Tm-Su. None of the strains was resistant to ciprofloxacin or ceftriazone (Table 1).

PFGE analysis. Stable and reproducible PFGE profiles were obtained when the analysis was repeated on three separate occasions (data not shown). The three most useful restriction endonucleases for *S. typhi* genome were *Xba*I, *Spe*I and *Avr*II. Our previous work showed that *Spe*I and *Avr*II do not necessarily increase discrimination (Thong *et al.*, 1994). Hence, only *Xba*I was used for further analysis. *Xba*I digestion of DNA from the 52 MDR strains from four different countries produced only eight different PFGE profiles (PFPs) consisting of 15 to 20 DNA fragments with sizes ranging 40 kb to 400 kb (F values ranged from 0.52 to 1.0)(Table 1, Figure 1). Among the six unrelated Malaysian strains, five PFPs, X1, X1a, X1c, X1d and X4 were observed. PFPs X1a, X1c and X1d differed from X1 by 1 to 2 bands (F=0.92). The 13 strains from Vietnam that were isolated from different parts of Vietnam between years 1996-1997 had only four closely related PFPs, X1 (n=10), X1a (n= 1), X1b (n=1) and X2 (n=1). Similarly, 96% (24/25) of the MDR strains from Pakistan were indistinguishable with a PFP X1. The 8 Indian strains were differentiated into four PFPs (F = 0.54-1.0). Overall, PFGE analysis showed that there was a remarkable homogeneity among the epidemiologically unrelated MDR *S.typhi* isolated at different times and from different geographical areas with at most, 2-3 DNA fragments difference (Figure 1). In contrast, PFGE was more useful in differentiating the drug sensitive than the MDR strains as the 11 strains could be subtyped into 9 PFPs (F = 0.50-1.0) (Table 1).

AFLP analysis. Genomic DNA from randomly selected 53 *S.typhi* strains were restricted with enzymes *Eco*RI and *Mse*I, followed by ligation of respective AFLP adaptors. The resultant materials were amplified with non-selective AFLP primers. A total of 8 selective primer combinations were used (Table 2). These primers combination were chosen arbitrarily and hence were unbiased for being particularly informative or uninformative. A total number of 596 fragments were generated, 44 of which were constant, 253 were parsimony-uninformative and 298 were parsimony-informative (Table 2). Assuming an average fragments length of 200 bp, the coverage was estimated to be approximately 120,000 bp or about 3% of the *S. typhi* genome.

A total of 16 and 5 AFLP profiles were obtained for the MDR and drug-sensitive strains respectively (Table 1). Profile A1 was the dominant pattern (30%) and was represented by strains from different countries (Table 1, Figure 2). MDR *S. typhi* strains from each country had unique profile: profiles A5 and A2a were exclusively found in the Malaysian strains, profiles A5a, A5b, A5c for Vietnam strains, profiles A6, A6a, A6b and A11 for the Indian strains and profiles A7, A7a, A8, A9, and A9a among the Pakistani strains (Table 1) Based on the combination of the entire AFLP database, a dendrogram was generated to ascertain the genetic relationship among the strains (Figure 2). The majority of the MDR strains from Pakistan were separated into many clusters comprising of strains that were every similar but not identical. MDR strains from different regions were closely related with one or two clusters consisting of strains from different countries (Figure 2). AFLP differentiated the 11 drug-sensitive strains into five profiles, A2, A2a, A3, A4, and A10.

AFLP with a DI of 0.98 was more discriminative than PFGE (DI=0.43) in subtyping the MDR *S. typhi*. For example, the 24 MDR Pakistani strains which shared a single PFP X1

Table 2. AFLP primer combinations used in this study and number of fragments

Primer Combination	No. of Fragments	No. of constant fragments	No. of uninformative fragments	No. of informative fragments
EcoR1-0/Mse1-CA	82	3	27	52
EcoR1-0/Mse1-CC	86	19	31	36
EcoR1-0/Mse1-CT	65	6	26	33
EcoR1-AA/Mse1-0	64	1	22	41
EcoR1-C/Mse1-G	69	4	32	33
EcoR1-C/Mse1-T	75	1	51	23
EcoR1-G/Mse1-G	46	3	21	22
EcoR1-G/Mse1-T	108	7	43	58
Total	**595**	**44**	**253**	**298**

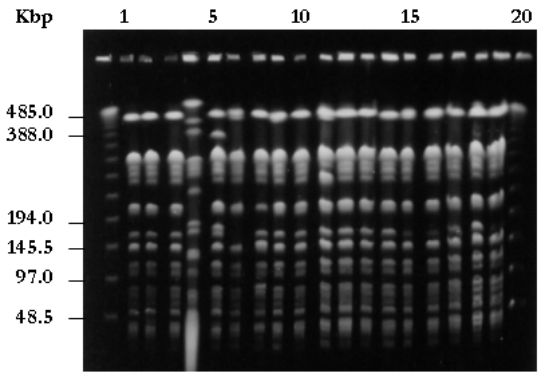

Figure 1. Representative DNA fingerprints of *Xba*I digested *Salmonella typhi* strains from Malaysia (TP), Pakistan (PR), Vietnam (Vn) and India (S) (profiles are indicated in parentheses). Lanes 1 through 20: Lambda DNA Standard, TP12 (X1), TP28 (X1), S5 (X1), S43 (X3), Vn243 (X2), TP30 (X1a), Vn138 (X1), Vn139 (X1), Vn181 (X1), Vn99 (X1b), PR5 (X1), PR48 (X1), PR36 (X1), PR8 (X1), S45 (X1), S23 (X1), Vn109 (X1), Vn97 (X1a), Lambda DNA standard.

profile could be subtyped into 8 AFLP patterns. However, for the analysis of the 11 drug-susceptible strains, PFGE was more discriminative (9 PFPs, DI =0.96) than AFLP (5 profiles, DI=0.53). Both AFLP and PFGE could differentiate the MDR from the drug-susceptible strains (Figure 2).

DISCUSSION

Typhoid fever remains an important public health problem in tropical countries and hence, it is important to characterize the causative agent, *S. typhi* in tracing an infection outbreak or detection of new virulent strain. Traditional characterization of *S. typhi* has relied on phage typing. However, phage types may not be a very useful epidemiological marker when a large number of strains belong to one or three phage types only. In the present study, phage typing has a very low discriminatory ability as 68% of the MDR strains tested belonged to group E1. This is in concordant to that reported by Hampton *et al.* (1998) that *S.typhi* strains originated from Southeast Asia are mostly E1 and M1. Therefore, phage typing is very often supplemented by more discriminative molecular based-techniques to improve strain differentiation.

Macrorestriction using PFGE has been reported to be the most useful and discriminative tool for the discrimination of even closely related bacterial isolates and is the current typing tool for analysis of *Salmonella* spp. (Hampton *et al.*, 1998). In our previous study, 50 MDR *S.typhi* strains from Pakistan were indistinguishable (Thong *et al.*, 2000). Here, we extend the PFGE analysis to 53 MDR isolates of *S. typhi* from four different Asian countries and eight profiles which differed in one to six bands were obtained. This observation was similar to the report by Mirza *et al.* (2001), where 5 different *Xba*I profiles were noted among the 194 MDR *S. typhi* from 6 different countries. However, the majority of the MDR strains (94%) examined had very similar PFGE patterns of one to three bands difference. Although the MDR *S.typhi* strains were widely disseminated among the Asian countries, considerable genetic homogeneity was demonstrated amongst these epidemiologically unrelated strains. When we compared the PFFs found in our study with others described in the literature and also generated by *Xba*I (Shanahan *et al.*, 1998, Kariuki *et al.*, 2000, Mirza *et al.*, 2000, Hampton *et al.,* 2000, Connerton *et al.*, 2000) it was observed that PFPs X1 and the subtypes were similar, although precise comparison may be difficult due to different running conditions and/or the evaluation criteria used by each author. The high prevalence of this genotype, and its widespread distribution in the various endemic countries suggest that it may be an epidemic clone among humans. Tenover *et al.* (1995) has published criteria relating to the restriction site variation in PFGE to epidemiological evidence of clonality. These criteria state that 2 to 3 PFGE fragments differences may be due to a single genetic event. The variant forms of the X1 profile could probably be associated with homologous rearrangement (Liu and Sanderson, 1995, Echeita and Usera, 1998).

In this study, PFGE is less useful in discriminating the MDR strains although it is still very useful in discerning the drug-susceptible strains where multiple subtypes exist. These multiple subtypes differed in more than 6 bands and could not be explained by just homologous rearrangements. Previous studies have shown that PFGE could differentiate closely related *S. typhi* and wide genetic variations exist among drug-susceptible strains (Thong *et al.*, 1994, 2002).

AFLP allows the differentiation of related bacterial species through comparison of complex banding patterns produced by PCR-amplified restriction fragments. The profiles generated by AFLP are highly reproducible because of stringent PCR conditions and the analysis can be completed in 2-3 working days (Vos *et al.*, 1995). In this study, MDR *S.typhi* genomic DNA was digested using two REs, *Mse*I and *Eco*RI and adapter DNA are ligated to fragments prior to PCR. AFLP markers are extremely sensitive to even small sequence variations, using PCR and high resolution electrophoresis to examine restriction fragments. AFLP fingerprinting has been used successfully to differentiate different *Salmonella* serovars (Aarts *et al.*, 1998, Lindstedt *et al.*, 2000), *S. typhimurium* (Tamada *et al.,* 2001), *S. enteritidis* (Desai *et al.*, 2001) and drug-sensitive strains of *S. typhi* (Nair *et al.*, 2000). In this study, AFLP was more discriminative than PFGE as it further subdivided the 8 *Xba*I profiles of the 52 MDR strains into 16 AFLP patterns. Strains with indistinguishable PFGE profiles had AFLP patterns which differed in 1 to 3 bands. These small differences may be consistent either with a genetic change (i.e. point mutation or an insertion or deletion of DNA) or with variation in

Figure 2. Dendrogram of a UPGMA Boostrap analysis (1000 replicates) of combined AFLP data of *Salmonella typhi* from different geographical areas in Asia. The numbers on the branches indicate confidence estimates (in %) of the particular cluster configuration. Clade information consists of strain ID (phage type/resistance phenotype/*Xba*I PFGE type) AFLP type.

DNA methlylation. Overall, cluster analysis indicated clonal nature of this particular clone of MDR *S.typhi* strains despite the minor variations. Cluster analysis based on the AFLP and PFGE data using unweighted pair group mean averages could differentiate MDR strains from different geographical region and that the drug-sensitive strains were in a distinct cluster. The clustering of MDR strains from each country and the presence of a dominant *Xba*I–PFGE pattern indicated that the MDR *S.typhi* had probably been spread clonally in these countries. AFLP is clearly more discriminative than PFGE in differentiating the MDR *S.typhi,* hence providing an alternative, sensitive method for detailed analysis of the multidrug resistant strains. It was noted that the AFLP patterns of the sensitive strains could also be differentiated by PFGE. Discrepancies like these might indicate that the strains in questions have no mutations within the *Mse*I and *Eco*RI restriction sites or no mutations in the sequence adjacent to the restriction sites which are complementary to the selective primer extensions, as compared to the polymorphisms within the *Xba*I sites.

Different typing methods assess different segments of the chromosome. Both AFLP and PFGE assess genetic diversity over the entire genome. However, PFGE is a macrorestriction analysis while AFLP detect mutation at restriction endonuclease cut sites. The status of a single fragment difference in AFLP is unlike that of a band shift in PFGE because an AFLP fragment is very much smaller, is precisely sized (±1bp), can be sequenced and mapped. Therefore, a single fragment difference in AFLP may defined a new strain.

The study reiterates the importance of using appropriate molecular typing tools in differentiating and subtyping bacterial pathogens. Even though PFGE has been shown to be the most discriminative tool for analysis of drug sensitive strains, it may not be discriminative enough to distinguish MDR *S. typhi* and another method, such as AFLP should be used concurrently. For the purpose of epidemiological surveillance, monitoring clonal spread and prevalence in populations over extended periods would require a 'library ' of typing systems (Struelens *et al.,* 1998). Such methods must be easily standardized, have a high throughput and reproducible. The issue of standardization is particularly important as it would enable results from different laboratories to be compared. Of the many typing systems, both PFGE and AFLP are particularly suitable as both generate reproducible results, applicable to many microorganisms and direct accumulative analysis is possible with appropriate software. Although AFLP is technically simpler, it is more costly than PFGE as it requires sophisticated algorithm and software to analyse the complex banding profiles.

In conclusion, AFLP is more discriminative than PFGE in distinguishing MDR *S.typhi* strains. Cluster analysis based on AFLP and PFGE could distinguish the MDR from drug sensitive *S. typhi* strains and these MDR S.typhi strains from various countries in this region were probably part of an endemic clone.

ACKNOWLEDGEMENTS

The authors thanked the Salmonella Reference Centre, Institute of Medical Research, Kuala Lumpur for the phage typing of strains. The work described was funded by IRPA Grant 06-02-03-0625, 06-02-03-750 and 06-02-03-1007 from the Ministry of Science, Technology & Environment, Malaysia. The authors thanked Dr Phung Dac Cam (National Institute of Hygiene and Epidemiology, Hanoi, Vietnam), and Dr Rama Chaudhry (All India Institute of Medical Sciences, New Dehli, India) for providing some of the strains for comparison.

REFERENCES

Aarts, H.J.M., Van Lith, L.A. J.T. and Keljer, J. 1998. High –resolution genotyping of *Salmoenlla* strains by AFLP-fingerprinting. *Letters Applied Microbiology* 26: 131-135.

Bhutta ZA. 1996. Impact of age and drug resistance on mortality in typhoid fever. *Archives of Diseases in Childhood* 75:214-217.

Connerton,P., Wain, J., Hien, T. T., Ali, T., Parry,C., Chinh,N.T., Vinh, H., Ho, V.A., Diep,T.S., Day, N.P.J., White, N.J., Dougan, G., and Farrar, J.J. 2000. Epidemic Typhoid in Vietnam: Molecular tying of multiple-antibiotic resistant *Salmonella enterica* serotype Typhi from four outbreaks. *Journal of Clinical Microbiology* 38: 895-897.

Desai, M., Threlfall, E.J., and Stanley, J. 2001. Fluorescent amplified fragment length polymorphism subtyping of *Salmonella enterica* serovar Enteritidis Phage Type 4 Clone complex. *Journal of Clinical Microbiology* 39: 210-206.

Echeita, M.A. and Usera, M.A. 1998. Chromosomal rearrangements in *Salmonella enterica* serotype Typhi affecting molecular typing in outbreak investigations. *Journal Clinical Microbiology* 36: 2123-2126.

Fica, A.E., Prat-Miranda, S., Fernandez-Ricci, A., D'Ottone, K., and Cabello, F.C. 1996. Epidemic typhoid in Chile: analysis by molecular and conventional methods of *Salmonella* Typhi strain diversity in epidemic (1977 and 1981) and nonepidemic (1990) years. *Journal of Clinical Microbiology* 34:1701-1707.

Hampton, M.D., Ward, L.R., Rowe, B., and Threlfall, J. 1998. Molecular fingerprinting of multidrug-resistant *Salmonella enterica* serotype Typhi. *Emerging Infectious Diseases* 4:317-320.

Hermans, P.W.M., Saha, S.K., van Leeuwen, W.J., Verbrugh, H.A., van Belkum, A., and Goessens, W.H.F. 1996 Molecular typing of *Salmonella typhi* strains from Dhaka (Bangladesh) and development of DNA probes identifying plasmid-encoded

multidrug-resistant isolates. *Journal of Clinical Microbiology* 34:1373-1379.

Hunter, P.R. and Gaston, M.A. 1988. Numerical index of the discriminatory ability of typing system: an application of Simpson' Index of diversity. *Journal Clinical microbiology* 26: 2465-2466.

Janssen, P., Coopman, R., Huys, G., Swings, J., Bleeker, M., Vos,P., Sabeau, M., and Kersters, K. 1996. Evaluation of the DNA fingerprinting method AFLP as a new tool in bacterial taxonomy. *Microbiology* 142: 1881-1893.

Kariuki, S., Gills, C, Revathi, G., and Hart, C.A. 2000. Genotypic analysis of multidrug resistant *Salmonella enterica* serovar Typhi, Kenya. *Emerging Infectious Diseases* 6: 649-651.

Lindstedt, B-A., Heir, E., Vardund, T., and Kapperud, G. 2000. Fluorescent amplified fragment length polymorphism genotyping of *Salmonella* subspecies *enterica* Serovars and comparison with pulsed field gel electrophoresis typing. *Journal of Clinical Microbiology* 38:1623-1627.

Ling, J.M., Lo, N.W.S., Ho, Y.M. Kam, K.M., Hoa, N.T.T., Phi, L.T., and Cheng, A.F. 2002. Molecular methods for the epidemiological typing of *Salmonella enterica* serotype Typhi from Hong Kong and Vietnam. *Journal of Clinical Microbiology* 36: 292-300.

Liu, S.L. and Sanderson, K.E. 1995. Rearrangements in the genome of the bacterium *Salmonella typhi*. *Proceeding of the National Academy Science USA* 92: 1018-1022.

Mirza, S., Kariuki, S., Mamun, K.Z., Beeching, N.J., and Hart, C.A. 2000. Analysis of plasmid and chromosomal DNA of multiresistant *Salmonella enterica* serovar Typhi from Asia. *Journal of Clinical Microbiol*ogy 38:895-897.

Nair, S., Schreiber, E., Thong, K.L. Pang, T. and Altwegg, M. 2000. Genotypic characterization of *Salmonella typhi* by amplified fragment length polymorphism fingerprinting provides increased discrimination as compared to pulsed field gel electrophoresis and ribotyping. *Journal of Microbiological Methods* 41: 35-43.

Navarro F., T. Llovet, M.A. Echeita, P. Coll, A. Aladuena, M. Usera, and Prats. G. 1996. Molecular typing of *Salmonella enterica* serovar Typhi. *Journal of Clinical Microbiology* 34:2831-2834.

Rowe, B., Ward, LR, and Threlfall, E.J. 1997. Multidrug-resistant *Salmonella* Typhi: a worldwide epidemic. *Clinical Infectious Diseases* 24 : 106-109.

Saito, H and Miura, K. 1963. Preparation of transforming deoxyribonucleic acid by phenol treatment. *Biochemistry Biophysical Acta* 72: 619-629.

Shanahan, P.M.A., Jesudason, M.V., Thomson, C.G., and Amyes, S.G.B. 1998. Molecular analysis and identification of antibiotic resistance genes in clinical isolates of *Salmonella typhi* from India. *Journal of Clinical Microbiology* 36:1595-6000.

Shangkuan,Y.H., and Lin, H.C. 1998. Application of random amplified polymorphic DNA analysis to differentiate strains of *Salmonella typhi* and other *Salmonella* species. *Journal of Applied Microbiology* 85: 693-702.

Struelens, M.J., Y. De Gheldre and Deplano. A. 1998. Comparative and library epidemiological typing systems: outbreak investigations versus surveillance systems. *Infection Control Hospital Epidemiology* 19: 565-59.

Tamada, Y., Nakaoka, Y., Nishimore, K., Doi, A., Kumaki, T., N. Uemura, Tanaka, K., Makion, C., Sameshima, T., Akiba, M. , Nakazawa, M. and Uchida, U. 2001. Molecular typing and epidemiological study of *Salmonella enterica* serotype Typhimurium isolates from cattle by fluorescent amplified-fragment length polymorphism fingerprinting and pulsed field gel electrophoresis. *Journal of Clinical Microbiology* 39: 1057-1066.

Tenover, F.C., Arbeit, R.D., and Goering, R.V. 1995. Interpreting chromosomal DNA restriction patterns produced by pulsed field gel electrophoresis : criteria for bacterial strain typing. *Journal of Clinical Microbiology* 33:2233-2239.

Thong, K.L., Cheong, Y.M., Puthucheary S.D., Koh C.L., and Pang, T. 1994. Epidemiologic analysis of sporadic and outbreak *Salmonella typhi* isolates by pulsed field gel electrophoresis. *Journal of Clinical Microbiology* 32:1135-1141.

Thong, K.L. and Pang, T.. 1996. A rapid, simplified method for preparation of chromosomal DNA from pathogenic bacteria for use in pulsed field gel electrophoresis. *Asia Pacific Journal Molecular Biology and Biotechnology* 4: 59-62.

Thong, K.L., Bhutta, Z.A., and Pang, T. 2000. Multidrug resistant *Salmonella enterica* serotype Typhi are genetically homogenous and coexist with antibiotic sensitive strains as distinct, independent clones. *International Journal of Infectious Disease* 4: 194-197.

Thong, K.L., Goh, Y.L., Yasin, R.M., Lau, M.G., Passey, M., Pang, T. and Reeder, J. 2002. The increasing diversity of *Salmonella* Typhi from Papua New Guinea. *Journal of Clinical Microbiolology* 40: 4156-4160.

Vos, P. Hogers, R., Bleeker, M., Reijans, M. Vande Lee, T., Hornes, M., Frijters, A., Pot, J., Peleman, J., Kuiper, M. and Zabeau, M. 1995. AFLP: a new technique for DNA fingerprinting. *Nucleic Acids Research* 23: 4407-4414.